

[Na] and [K] Dependence of the Na/K Pump Current-Voltage Relationship in Guinea Pig Ventricular Myocytes

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ABSTRACT Na/K pump current was determined between -140 and $+60$ mV as steady-state, strophanthidin-sensitive, whole-cell current in guinea pig ventricular myocytes, voltage-clamped and internally dialyzed via wide-tipped pipettes. Solutions were designed to minimize all other components of membrane current. A device for exchanging the solution inside the pipette permitted investigation of Na/K pump current-voltage (I-V) relationships at several levels of pipette [Na] ($[Na]_{pip}$) in a single cell; the effects of changes in external [Na] ($[Na]_o$) or external [K] ($[K]_o$) were also studied. At 50 mM $[Na]_{pip}$, 5.4 mM $[K]_o$, and ~ 150 mM $[Na]_o$, Na/K pump current was steeply voltage dependent at negative potentials but was approximately constant at positive potentials. Under those conditions, reduction of $[Na]_o$ enhanced pump current at negative potentials but had little effect at positive potentials: at zero $[Na]_o$, pump current was only weakly voltage dependent. At 5.4 mM $[K]_o$ and ~ 150 mM $[Na]_o$, reduction of $[Na]_{pip}$ from 50 mM scaled down the sigmoid pump I-V relationship and shifted it slightly to the right (toward more positive potentials). Pump current at 0 mV was activated by $[Na]_{pip}$ according to the Hill equation with best-fit $K_{0.5} \cong 11$ mM and Hill coefficient $n_H \cong 1.4$. At zero $[Na]_o$, reduction of $[Na]_{pip}$ seemed to simply scale down the relatively flat pump I-V relationship: Hill fit parameters for pump activation by $[Na]_{pip}$ at 0 mV were $K_{0.5} \cong 10$ mM, $n_H \cong 1.4$. At 50 mM $[Na]_{pip}$ and high $[Na]_o$, reduction of $[K]_o$ from 5.4 mM scaled down the sigmoid I-V relationship and shifted it slightly to the right: at 0 mV, $K_{0.5} \cong 1.5$ mM and $n_H \cong 1.0$. At zero $[Na]_o$, lowering $[K]_o$ simply scaled down the flat pump I-V relationships yielding, at 0 mV, $K_{0.5} \cong 0.2$ mM, $n_H \cong 1.1$. The voltage-independent activation of Na/K pump current by both intracellular Na ions and extracellular K ions, at zero $[Na]_o$, suggests that neither ion binds within the membrane field. Extracellular Na ions, however, seem to have both a voltage-dependent and a voltage-independent influence on the Na/K pump: they inhibit outward Na/K pump current in a strongly voltage-dependent fashion, with higher apparent affinity at more negative potentials ($K_{0.5} \cong 90$ mM at -120 mV, and ~ 170 mM at -80 mV), and they compete with extracellular K ions in a seemingly voltage-independent manner. Possibly, Na ions are released from the Na/K

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pump to the exterior in two stages, one involving charge translocation and the second involving a subsequent voltage-insensitive competition with external K ions.

INTRODUCTION

That the turnover rate of the Na/K pump varies with membrane potential is no longer in dispute (for review, see De Weer et al., 1988), but the precise form of that voltage dependence is. In the preceding paper (Gadsby and Nakao, 1989), the Na/K pump I-V relationship of guinea pig ventricular myocytes was demonstrated to have a monotonic, sigmoid shape when determined under conditions in which the pump was strongly activated, but in which contamination from changes in nonpump currents (due, e.g., to pump-mediated shifts of [K] or [Na], just outside or inside the cell, respectively) was shown to be negligible. The sigmoid shape is readily accounted for by assuming that the Na/K pump transport cycle includes only a single charge-translocating, and hence voltage-dependent, step (Hansen et al., 1981) which, however, is not itself rate limiting in the cycle but instead controls the level of enzyme intermediate entering the rate-limiting, though voltage-insensitive step (Bahinski et al., 1988). On the other hand, cardiotoxic steroid-sensitive I-V relationships determined in *Xenopus* oocytes have sometimes (Lafaie and Schwarz, 1986; Schweigert et al., 1988), but not always (Rakowski and Paxson, 1988), included a region of steep negative slope at positive membrane potentials. The negative slope has been ascribed to a property of the Na/K pump and interpreted as signaling the existence of an additional voltage-sensitive step which involves charge movement in the opposite direction through the membrane field (Lafaie and Schwarz, 1986; Schweigert et al., 1988).

The aim of the experiments presented here was to reliably determine steady-state Na/K pump I-V relationships over an extensive range of pump activation, obtained by varying intracellular [Na] or extracellular [K], and over an additional range of driving forces established by varying extracellular [Na]. One of the goals was to see how the shape of the pump I-V curve was altered and, in particular, to see whether a negative slope could be detected under any of those widely disparate conditions. But the major goal was to determine the kinetics of pump activation by internal Na and external K and to learn whether, and to what extent, they are influenced by membrane potential. Using intracellular and extracellular solutions that minimized other membrane currents, and a simple technique for changing the pipette solution during whole-cell current recording (Soejima and Noma, 1984), Na/K pump current, measured as strophanthidin-sensitive current, could be obtained under several different conditions in the same cell. We found no consistent evidence for a negative slope in the pump I-V relationship but found that, at a fixed membrane potential, pump current was a saturable function of both intracellular [Na] and extracellular [K], and that pump activation by those ions was only weakly affected by changes of membrane potential. Extracellular [Na], however, strongly influenced the voltage dependence of Na/K pump current.

Some preliminary results were communicated while the work was in progress (Nakao and Gadsby, 1985; Gadsby and Nakao 1986, 1987*a* and *b*).

MATERIALS AND METHODS

The methods used for these experiments are fully described in the preceding paper (Gadsby and Nakao, 1989). A few pertinent details are repeated here.

The modified, nominally Ca-free Tyrode's solution used in most experiments to establish control conditions contained (in millimolar): 145 NaCl, 5.4 KCl, 2.3 MgCl₂, 2 BaCl₂, 0.2 CdCl₂, 5.5 dextrose, 5 Hepes/NaOH (pH 7.4). The K concentration was varied between 0 and 10 mM by replacing KCl with NaCl or vice versa. Corresponding Na-free external solutions were made by replacing all NaCl with *N*-methyl-D-glucamine (NMG) chloride and NaOH with NMG base. Appropriate mixtures of the ~150 mM Na and Na-free solutions gave intermediate levels of [Na]. All external solutions were warmed but not oxygenated; experiments were carried out at ~36°C.

All pipette solutions were K-free. The Na-free pipette solution used to begin each experiment contained (in millimolar): ~130 CsOH, 100 aspartic acid, 5 pyruvic acid, 20 TEACl, 3 MgCl₂, 10 EGTA, 10 MgATP, 5 Tris₂-creatine phosphate, 5.5 dextrose, 10 Hepes (pH 7.4). In another pipette solution 100 mM NaOH replaced the same amount of CsOH, so that [Na]_{pip} could be varied between 0 and 100 mM by mixing the two solutions.

Strophanthidin was kept as a 0.5 M stock solution in dimethylsulfoxide (DMSO) and was routinely used at a concentration of 0.5 mM in ~150 mM Na solutions, but at 2 mM whenever [Na]_o was <150 mM. Control tests showed that up to 0.5% DMSO was without effect on membrane current under these conditions (Gadsby and Nakao, 1989). Moreover, because a series of control experiments demonstrated that under our experimental conditions strophanthidin-sensitive current is virtually identical to Na/K pump current and, in particular, is practically free of contaminant components reflecting changes in [K] or [Na] near the cell membrane on stopping the pump (Gadsby and Nakao, 1989), "strophanthidin-sensitive" and "Na/K pump" will be used interchangeably when referring to those I-V relationships obtained by subtraction.

Whole-cell currents were elicited by 100-ms voltage pulses, and both signals were filtered at 2 kHz and digitized on-line at 6 kHz and stored in a microcomputer for later analysis; data acquisition and analysis were controlled via ASYST software. Steady-state current and voltage levels were obtained by averaging points over 30 ms near the end of each pulse.

The above conditions were modified in two instances: (a) In our early experiments (Gadsby et al., 1985; Nakao and Gadsby, 1985; Gadsby and Nakao, 1986) external solutions contained 0.9 mM Ba (instead of 2 mM), sufficient to markedly suppress, but not abolish, the sensitivity of background membrane current to extreme [K] changes (Gadsby and Nakao, 1989). Nevertheless, pump-mediated changes in extracellular [K] were probably far too small to affect background current, so that strophanthidin-sensitive current remained free of such contamination. However, in a few experiments reported here with cells exposed to Na-free solutions, external K was deliberately withdrawn when 0.5 mM strophanthidin was applied, to ensure full pump inhibition (see e.g., Fig. 6, below). Although the difference current is expected to yield an accurate measure of pump current at potentials positive to -40 mV, even in those circumstances (Gadsby and Nakao, 1989), the difference I-V relationship at more negative potentials is contaminated by a small [K]_o-dependent nonpump current. Accordingly, all I-V relationships from those experiments have been discarded, and only pump current levels at 0 mV, at various [Na]_{pip}, were retained for inclusion with the more recent data obtained using 2 mM Ba and constant [K]_o. (b) Four of the eight experiments to determine the influence of [Na]_{pip} on pump current at 150 mM [Na]_o were carried out in the presence of 0.1 mM LaCl₃ (e.g., Fig. 4, below), which was found to reduce leakage conductance (roughly voltage independent) and low-frequency background current noise, possibly by stabilizing the seal between pipette tip and cell membrane, and to thereby enhance accuracy of those pump current measurements.

A variable, slow (time constant ~ 20 min) rundown of Na/K pump current to about half its initial amplitude occurred in these experiments, presumably as a result of dialysis of the cell contents via the wide-tipped (~ 5 μm diameter) pipettes (Gadsby and Nakao, 1989). Because of this rundown, measurements of Na/K pump I-V relationships under test conditions (various $[\text{Na}]_{\text{pip}}$ or $[\text{K}]_o$) were usually interposed between two control measurements, which were subsequently averaged (weighted if the two time intervals differed by $>50\%$) before quantitative comparison with the test data.

Mean values are given \pm SEM unless otherwise indicated.

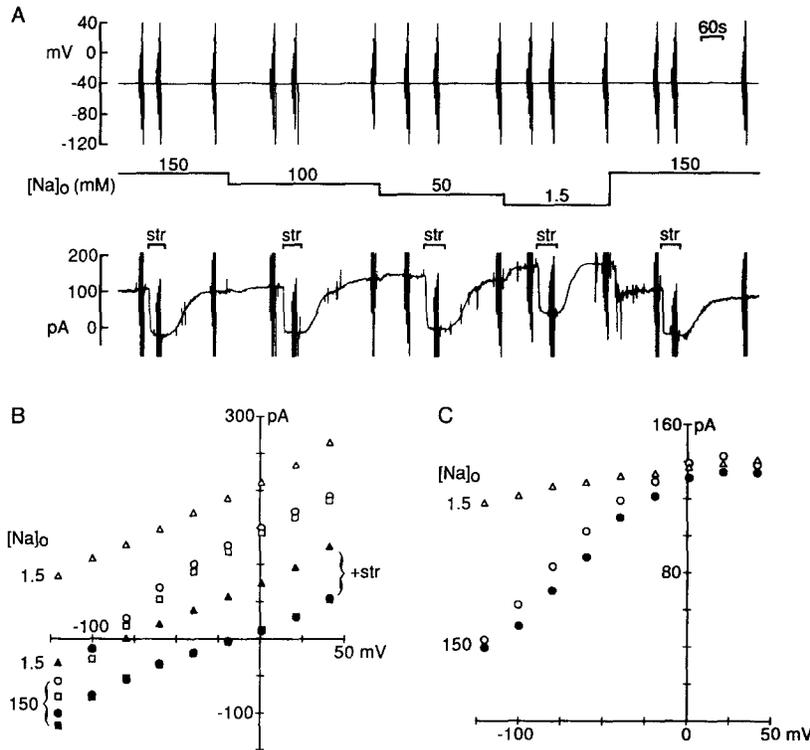


FIGURE 1. Influence of extracellular $[\text{Na}]$ on whole-cell currents in the presence and absence of 2 mM strophanthidin. (A) Chart records of membrane potential (*upper trace*) and cell current (*lower trace*): Groups of vertical lines show application of 100-ms voltage pulses (*upper*) to potentials between +40 mV and -120 mV (in 20-mV increments), and the resulting current changes (*lower*). Middle line indicates changes of $[\text{Na}]_o$; bars over the current record mark periods of exposure to strophanthidin (*str*). (B) Steady-state whole-cell I-V relationships from A, determined in the absence (*open symbols*) or presence (*solid symbols*) of strophanthidin; open symbols represent averages of the values obtained before and after each brief exposure to strophanthidin. For clarity, only results at 150 and 1.5 mM $[\text{Na}]_o$ are plotted; circles and squares show 150 mM $[\text{Na}]_o$ data before and after, respectively, the episode at 1.5 mM $[\text{Na}]_o$. (C) Steady-state strophanthidin-sensitive (i.e., Na/K pump) I-V relationships, at 150 mM $[\text{Na}]_o$ before (\circ) and after (\bullet) exposure to 1.5 mM $[\text{Na}]_o$ (Δ), obtained by appropriate subtraction of data in B, i.e., of the steady current levels in the presence of strophanthidin from the averages of those in its absence. $[\text{Na}]_{\text{pip}}$, 50 mM; $[\text{K}]_o$, 5.4 mM; cell capacitance, 177 pF; initial pipette resistance, 1.1 M Ω .

RESULTS

Influence of Extracellular [Na]

The chart recording in Fig. 1 A shows that in a cell equilibrated with 50 mM Na pipette solution, stepwise reduction of $[Na]_o$ from 150 to 1.5 mM caused a progressive outward shift of the holding current at -40 mV, in the presence of 2 mM strophanthidin as well as in its absence, which was fully reversed on reexposure to 150 mM $[Na]_o$. The current shift was a little smaller in the presence of the steroid, indicating that strophanthidin-sensitive current at -40 mV was somewhat larger during exposure to low $[Na]_o$. Representative whole-cell I-V relationships from this experiment, with and without strophanthidin, both at 1.5 mM $[Na]_o$ (triangles) and at 150 mM $[Na]_o$ (circles and squares), are presented in Fig. 1 B. In the absence of

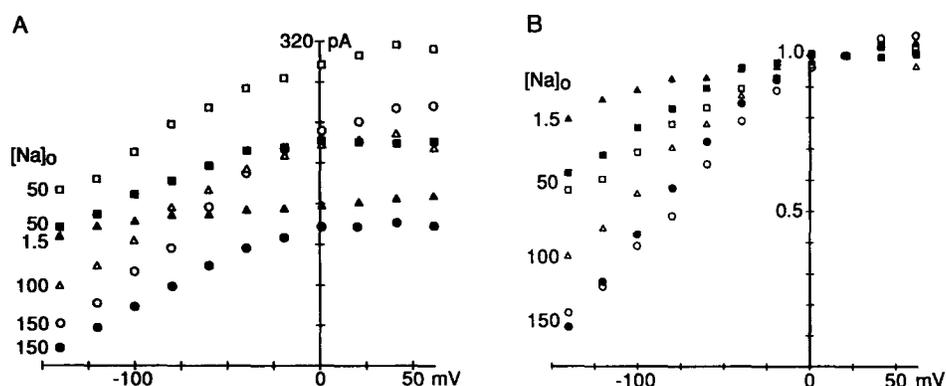


FIGURE 2. Influence of extracellular $[Na]$ on the Na/K pump I-V relationship. (A) Steady-state strophanthidin-sensitive I-V curves determined in a single cell, at the levels of $[Na]_o$ indicated, as described for Fig. 1. $[Na]_o$ was varied in the sequence: 50 mM (\square), 150 mM (\circ), 50 mM (\blacksquare), 100 mM (\triangle), 1.5 mM (\blacktriangle), 150 mM (\bullet); pump current amplitude at $+60$ mV declined in the same sequence, presumably due to rundown, as expected if $[Na]_o$ has little influence on pump current at positive potentials. (B) Same data (and same symbols) as in A. To allow for rundown, we normalized each I-V curve to its amplitude at $+20$ mV. $[Na]_{pip}$, 50 mM; $[K]_o$, 5.4 mM; cell capacitance, 183 pF; initial pipette resistance, 1.0 M Ω .

strophanthidin (open symbols), the outward current shift caused by lowering $[Na]_o$ was considerably larger at negative than at positive membrane potentials whereas, in the presence of strophanthidin (solid symbols), that current shift was roughly voltage independent. This implies a change in the shape of the strophanthidin-sensitive I-V relationship on lowering $[Na]_o$, with a relative increase in Na/K pump current at negative potentials, as demonstrated directly in Fig. 1 C. Because pump current was apparently unchanged at positive potentials, the effect of lowering $[Na]_o$ 100-fold was to greatly diminish the voltage dependence of pump current. At the intermediate levels of 50 and 100 mM $[Na]_o$, more moderate effects were observed in this cell, like those illustrated in Figs. 2 and 3.

Fig. 2 A shows Na/K pump I-V relationships determined in another cell exposed to 150, 100, 50, and 1.5 mM $[Na]_o$. To facilitate comparison, allowance was made for the gradual rundown of pump current in this experiment by normalizing each

pump I-V relationship with respect to its size at +20 mV, because pump current amplitude was approximately constant between +20 and +60 mV at all levels of $[\text{Na}]_o$. The resulting normalized curves are displayed in Fig. 2 B. They show that stepwise lowering of $[\text{Na}]_o$ causes, reversibly, a graded reduction in the voltage dependence of Na/K pump current between -140 and +60 mV. This result is confirmed by the averaged data gathered from nine cells, plotted in Fig. 3.

Since pump current apparently approaches a plateau level at positive potentials (Gadsby et al., 1985; Gadsby and Nakao, 1987a, 1989) that seems unaffected by changes in $[\text{Na}]_o$ (Fig. 1 C), the data were summarized by normalizing each pump

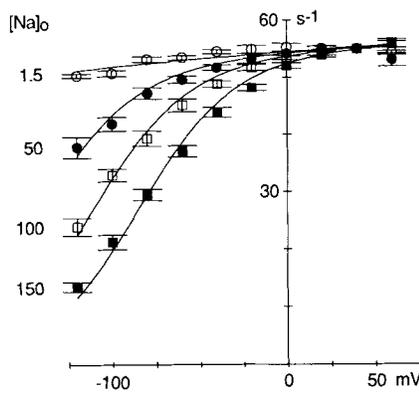


FIGURE 3. Graded influence of $[\text{Na}]_o$ on the shape of the Na/K pump I-V relationship at 50 mM $[\text{Na}]_{pip}$ and 5.4 mM $[\text{K}]_o$. Results from nine cells are summarized. Each steady-state Na/K pump I-V curve was normalized by dividing by its amplitude at +40 mV, and the resulting data at a given $[\text{Na}]_o$ were averaged. The mean normalized currents were then scaled by the average turnover rate, 55 s^{-1} (at +40 mV and 150 mM $[\text{Na}]_o$; Gadsby and Nakao, 1989) and the mean (\pm SEM) rates at the four $[\text{Na}]_o$ concentrations were plotted against pulse potential. The points at 1.5 mM $[\text{Na}]_o$ (O) represent five curves from five cells;

50 mM $[\text{Na}]_o$ (●), seven curves from seven cells; 100 mM $[\text{Na}]_o$ (□), 10 curves from eight cells; 150 mM $[\text{Na}]_o$ (■), 15 curves from nine cells. Overall, pump current at positive potentials showed no consistent variation with $[\text{Na}]_o$ (see Figs. 1 and 2), although it was possibly slightly depressed in two of five cells and apparently depressed (by ~20%) in one of five cells at 1.5 mM $[\text{Na}]_o$, but was possibly slightly enhanced in three of seven cells at 50 mM $[\text{Na}]_o$. The curves through the points obey: $\text{rate} = (\alpha c - \beta d) / (\alpha + \beta + c + d)$, where c, d are voltage-independent rate constants, $\alpha = \alpha^0 \cdot \exp[\delta VF/RT]$, $\beta = \beta^0 \cdot \exp[-(1 - \delta) VF/RT]$, and α^0 and β^0 indicate values of the voltage-sensitive rate constants of 0 mV. The curves show that the effect of lowering $[\text{Na}]_o$ can be mimicked by a progressive reduction of β , the backward voltage-sensitive rate constant of a pseudo two-state kinetic scheme. With β^0 and d fixed at 0 s^{-1} , and δ fixed at 0.1, best-fit values for α^0 and c were 490 and 61 s^{-1} , respectively, for the data at 1.5 mM $[\text{Na}]_o$. Then, holding all parameters but β^0 constant, curves were fitted to the data at 50, 100, and 150 mM $[\text{Na}]_o$, yielding values for β^0 of 3, 8, and 22 s^{-1} , respectively.

I-V relationship by its amplitude at +40 mV, then averaging results for each of the four levels of $[\text{Na}]_o$ tested, and finally scaling the mean normalized values by 55 s^{-1} , the estimated turnover rate at +40 mV (Gadsby and Nakao, 1989). One way of viewing the data in Fig. 3 is as an apparent voltage-dependent inhibition of outward Na/K pump current by extracellular Na ions, that inhibition being stronger at more negative potentials. Thus, inspection of Fig. 3 reveals that 150 mM $[\text{Na}]_o$ diminishes the outward pump current seen in the virtual absence of $[\text{Na}]_o$ (I-V curves at 0 mM and 1.5 mM $[\text{Na}]_o$ are indistinguishable; cf. Figs. 7 and 10) by some 70% at -120 mV, ~50% at -80 mV, ~20% at -40 mV, and by only ~5% at 0 mV. Despite the limited range of the data and their scatter, further analysis of this inhibition by

extracellular Na of the average pump current obtained at 1.5 mM $[Na]_o$ yields sigmoid inhibition curves at each voltage. The best fit parameters at -120 , -80 , and -40 mV were, respectively, 91 ± 2 , 170 ± 7 , and 357 ± 55 mM for the apparent $K_{0.5}$ for inhibition by $[Na]_o$, and 1.9 ± 0.1 , 1.8 ± 0.1 , and 1.6 ± 0.2 for the Hill coefficient: the $K_{0.5}$ changed e -fold in roughly 60 mV over this voltage range.

A voltage-dependent influence of $[Na]_o$ might be expected to occur if the backward voltage-sensitive rate constant (β) of the pseudo two-state kinetic cycle were a function of $[Na]_o$. Indeed, the curves drawn through the points in Fig. 3 confirm that the effect of lowering $[Na]_o$ can be mimicked by reducing β^0 while keeping all other model parameters constant. Possible interpretations of these results will be considered in the Discussion.

Influence of Intracellular [Na] at High [Na]_o

The influence of intracellular $[Na]$ was investigated by switching between pipette solutions with different $[Na]$. The standard concentrations was chosen to be 50 mM, and determination of the pump I-V relationship at any other pipette $[Na]$ was preceded and followed by control runs with 50 mM $[Na]_{pip}$, whenever possible, to make allowance for the variable rundown of pump current.

The chart record in Fig. 4 A is from an experiment to examine the effects of moderately low $[Na]_{pip}$. Switching from 50 to 8 mM $[Na]_{pip}$ caused an inward shift of holding current, with a half time of about 1 min, which was rapidly reversed on switching back to 50 mM $[Na]_{pip}$. Subsequent reduction of $[Na]_{pip}$ to 3 mM resulted in a somewhat greater inward shift of holding current, also half complete in ~ 1 min. These large current shifts reflect predominantly changes in Na/K pump current, because the holding current in the presence of strophanthidin remained practically unaltered throughout the experiment.

Representative steady-state, whole-cell I-V relationships from this experiment, with and without strophanthidin, are presented in Fig. 4 B, and the corresponding Na/K pump I-V curves, obtained by subtraction, are shown in Fig. 4 C. The curves determined at 50 mM $[Na]_{pip}$ had the relatively large amplitude and characteristic sigmoid shape already described for cells exposed to 150 mM external $[Na]$, and that approximate shape seemed to be retained at both 8 mM and 3 mM $[Na]_{pip}$. However, on lowering $[Na]_{pip}$ from 50 mM the pump I-V curves were not simply scaled down. This is shown by the normalized pump I-V relationships (Fig. 4 D) which were normalized, for each $[Na]_{pip}$, to the pump current amplitude at $+40$ mV, since that seemed to represent an approximately saturating (i.e., voltage-independent) level. The normalized curves reveal that lowering $[Na]_{pip}$ from 50 mM scaled down the pump I-V relationship and shifted it along the voltage axis to more positive potentials, in this instance by ~ 40 mV at 8 mM, and by some 50–60 mV at 3 mM $[Na]_{pip}$.

The effect of a more modest reduction of $[Na]_{pip}$, from 50 to 25 mM, is shown in Fig. 5. The chart record (Fig. 5 A) illustrates the resulting reversible shifts of holding current, again occurring with half-times of ~ 1 min, and indicates that strophanthidin-sensitive current at -40 mV was diminished only $\sim 15\%$ by this twofold reduction of $[Na]_{pip}$. The two pump I-V relationships determined at 50 mM $[Na]_{pip}$, that at 25 mM $[Na]_{pip}$, and a subsequent one at 3 mM $[Na]_{pip}$, all obtained by appro-

appropriate subtraction, and displaying the same characteristic shape, are presented in Fig. 5 B. Fig. 5 C shows the corresponding, normalized pump I-V relationships, each curve again obtained by normalizing pump current to its amplitude at +40 mV. Whereas little or no shift along the voltage axis is discernible for the reduction of $[\text{Na}]_{\text{pip}}$ from 50 to 25 mM, the further reduction to 3 mM seemed to cause a positive shift of ~ 55 mV (similar to that in Fig. 4 D).

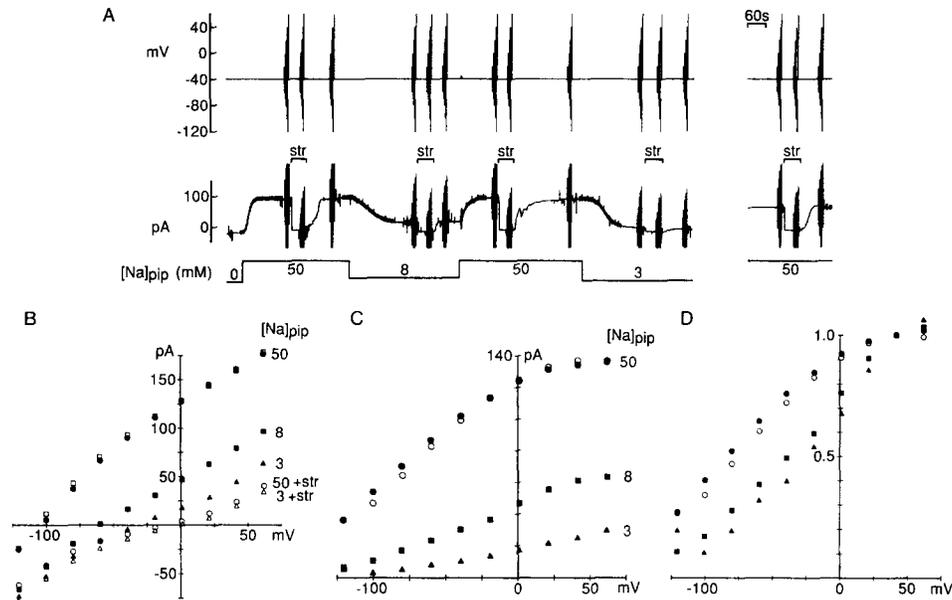


FIGURE 4. Influence of $[\text{Na}]_{\text{pip}}$ on whole-cell currents. (A) Chart records of voltage and current as in Fig. 1; the lower line indicates the approximate timing of changes in $[\text{Na}]_{\text{pip}}$, and the bars (*str*) mark exposures to 2 mM strophanthidin. The thickening of the current trace around each $[\text{Na}]_{\text{pip}}$ change results from repeated application (5 Hz) of brief (10 ms) 1-mV hyperpolarizing pulses, used to generate capacity currents as a monitor of the access resistance between the pipette and cell interior; the gap in the traces indicates omission of ~ 9 min of records. (B) Representative steady-state whole-cell I-V relationships from A, with and without strophanthidin and at different $[\text{Na}]_{\text{pip}}$ as indicated: for clarity, in the presence of strophanthidin, only the first curve at 50 mM $[\text{Na}]_{\text{pip}}$ and that at 3 mM $[\text{Na}]_{\text{pip}}$ are illustrated; the curves without strophanthidin are averages as in Fig. 1; at 50 mM $[\text{Na}]_{\text{pip}}$, only the first (\bullet) and second (\square) trial are depicted. (C) Na/K pump I-V relationships from A and B for 3 mM (\blacktriangle), 8 mM (\blacksquare), and 50 mM $[\text{Na}]_{\text{pip}}$ (first run, \circ ; second \bullet), obtained by appropriate subtraction as in Fig. 1. (D) Same data (and same symbols) as in C, but replotted after normalizing each I-V curve by its amplitude at +40 mV. $[\text{Na}]_{\text{o}}$, 150 mM; $[\text{K}]_{\text{o}}$, 5.4 mM; external solutions contained 0.1 mM LaCl_3 , found to diminish leak conductance apparently without affecting Na/K pump current; cell capacitance, 150 pF; initial pipette resistance, 1.3 M Ω .

In the four experiments that yielded extensive pump I-V relationships under these conditions at several levels of $[\text{Na}]_{\text{pip}}$, each successfully bracketed by control data at 50 mM $[\text{Na}]_{\text{pip}}$, the average (\pm SD) rightward shift of the normalized pump I-V curve (measured at half amplitude) on lowering $[\text{Na}]_{\text{pip}}$ from 50 mM amounted to 33 ± 8 mV at 8 mM, and 63 ± 7 mV at 3 mM $[\text{Na}]_{\text{pip}}$. These consistently observed

shifts of the pump I-V relationships are in the same direction as, but are far smaller than, the expected shifts of $E_{Na/K}$ on lowering intracellular [Na] from 50 mM to 25, 8, or 3 mM which, for comparison, are approximately +55, +147, and +225 mV, respectively.

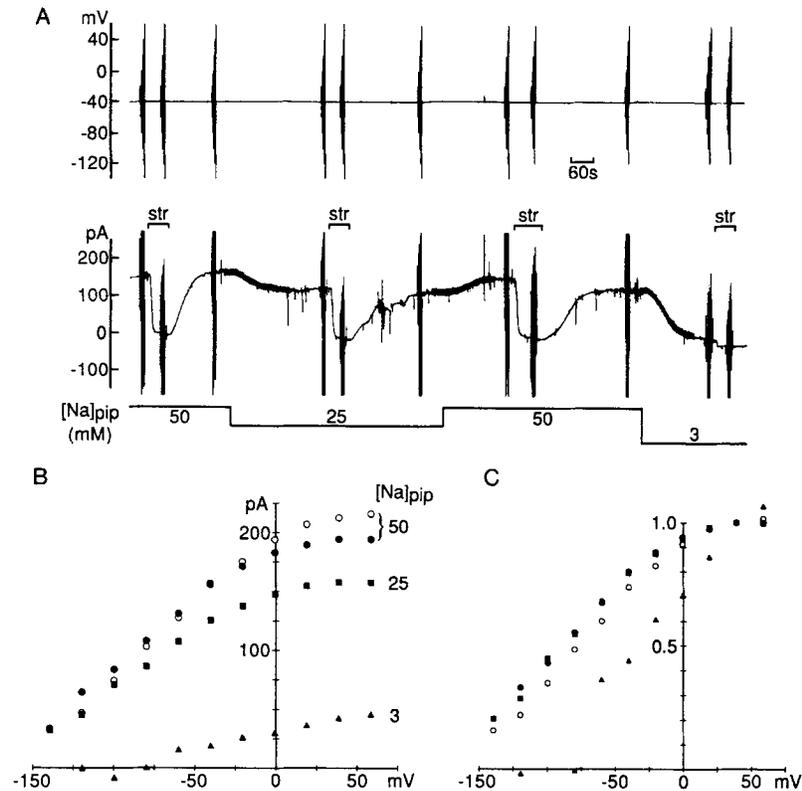


FIGURE 5. Effect of moderate reduction of $[Na]_{pip}$ on Na/K pump current. (A) Chart recording as in Fig. 4; strophanthidin concentration was 0.5 mM. (B) Steady-state strophanthidin-sensitive I-V relationships at 3 mM (\blacktriangle), 25 mM (\blacksquare), 50 mM $[Na]_{pip}$ (first run, \circ ; second \bullet) obtained by subtraction as in Fig. 1, but for later runs, at 50 mM and 3 mM $[Na]_{pip}$, strophanthidin I-V data were subtracted from corresponding I-V data determined immediately before exposure to strophanthidin. (The zero, or negative, current values at potentials ≤ -80 mV at 3 mM $[Na]_{pip}$ reflect instability of the whole-cell currents recorded at those voltages during that final exposure to strophanthidin, as well as the difficulty of accurately determining small differences between pairs of large numbers). (C) Same I-V curves, with same symbols, as in B, but after normalizing each curve to its amplitude at +40 mV. $[Na]_o$, 150 mM; $[K]_o$, 5.4 mM; cell capacitance, 191 pF; initial pipette resistance, 1.1 M Ω .

The data in Figs. 4 and 5 demonstrate that, when $[Na]_o$ is 150 mM, the voltage dependence of Na/K pump current (i.e., overall shape of the I-V curve) is conserved over a wide range of pump activation by intracellular [Na], but the sensitivity of the pump to $[Na]_{pip}$ seems to depend on membrane potential, and is somewhat greater at more positive potentials.

Influence of Intracellular [Na] at Zero [Na]_o

The chart records in Fig. 6 show representative changes in holding current (and in strophanthidin-sensitive current) at -40 mV, caused by varying $[\text{Na}]_{\text{pip}}$ in two cells exposed to Na-free external solution. The simultaneous withdrawal of extracellular

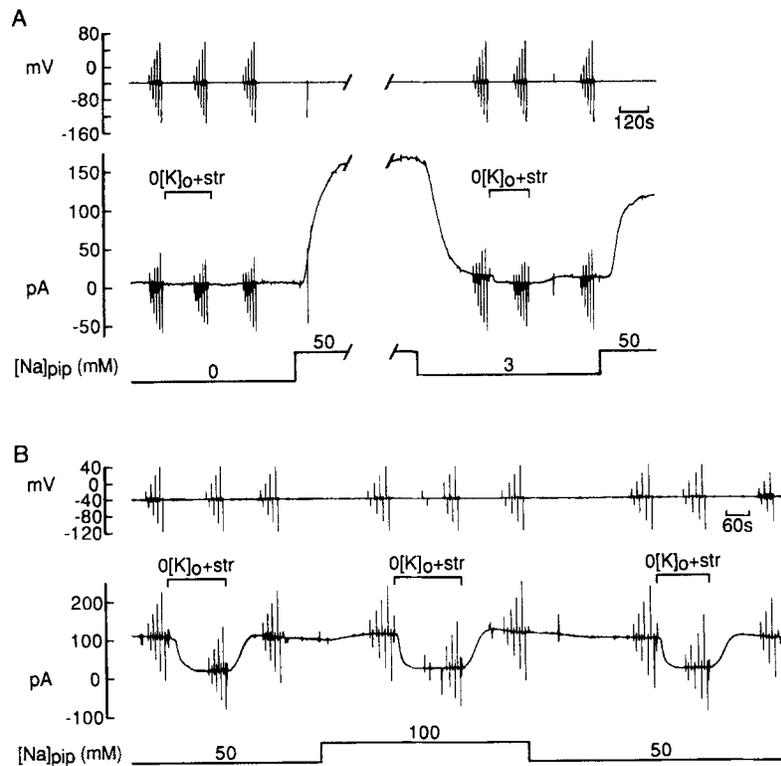


FIGURE 6. Influence of $[\text{Na}]_{\text{pip}}$ on whole-cell current at zero $[\text{Na}]_{\text{o}}$. (A and B) Chart records as in Fig. 4; bars over the current traces mark periods of pump inhibition with K-free solution containing 0.5 mM strophanthidin; $[\text{K}]_{\text{o}}$ at all other times, 5.4 mM. External solutions contained 0.9 mM Ba in these early zero $[\text{Na}]_{\text{o}}$ experiments (instead of the 2 mM Ba used in all later experiments) so that removing K_{o} abolished remaining inward K current at large negative potentials, but had no effect on nonpump current positive to -40 mV (Gadsby and Nakao, 1989). Although the chart pen does not faithfully record the currents elicited by the 100-ms voltage pulses, an effect of this kind is discernible in A, at 0 mM $[\text{Na}]_{\text{pip}}$ (when pump current should have been negligibly small), where exposure to zero $[\text{K}]_{\text{o}}$ plus strophanthidin can be seen to have caused, reversibly, small outward current shifts at potentials ≤ 100 mV. Initial pipette resistances, 1.4 M Ω (A), 1.0 M Ω (B).

K during applications of strophanthidin did not affect determination of pump current by subtraction at -40 mV or at more positive potentials. But, because the Na-free external solutions contained only 0.9 mM Ba in some of these early experiments (see, e.g., Gadsby et al., 1985; Gadsby and Nakao, 1986) removal of external K did elicit a small outward shift of background current at large negative potentials

(see Fig. 5 A of Gadsby and Nakao, 1989), causing the difference current to slightly underestimate pump current at those voltages.

The extents and rates of the holding current changes seen on varying $[Na]_{pip}$ are similar to those just described for cells exposed to 150 mM $[Na]_o$, and strophanthidin-sensitive current at -40 mV appears to be a saturable function of $[Na]_{pip}$, increasing very little on raising $[Na]_{pip}$ from 50 to 100 mM (Fig. 6 B), and declining to only a few percent of that maximum level on switching to 3 mM $[Na]_{pip}$ (Fig. 6 A). Fig. 6 A provides a clear demonstration of the ability of the present technique to detect the influence on the Na/K pump of only 3 mM $[Na]_{pip}$, in contradistinction to

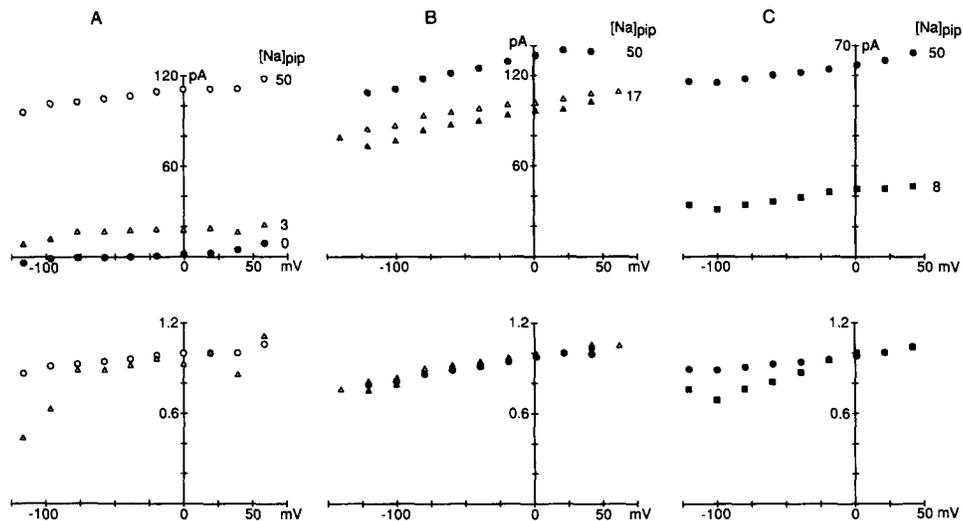


FIGURE 7. Influence of $[Na]_{pip}$ on steady-state Na/K pump I-V relationships at zero $[Na]_o$, 5.4 mM $[K]_o$ (and 2 mM Ba) throughout. Upper graphs show strophanthidin-sensitive I-V curves at the levels of $[Na]_{pip}$ indicated, obtained by subtracting I-V data recorded in the presence of 2 mM strophanthidin from that recorded just before its application (B, 17 mM $[Na]_{pip}$; C, 8 mM $[Na]_{pip}$) or after washing it off (B and C, 50 mM $[Na]_{pip}$), or from their average (A, 0, 3, and 50 mM $[Na]_{pip}$). Lower graphs show the same I-V relationships (same symbols) after normalizing each to its amplitude to +20 mV. (A) $[Na]_{pip}$ sequence: 0 mM, 50 mM, 3 mM; cell capacitance, 165 pF; initial pipette resistance, 1.1 M Ω . (B) $[Na]_{pip}$ sequence: 17 mM, 50 mM, 17 mM; cell capacitance, 126 pF; initial pipette resistance, 1.0 M Ω . (C) $[Na]_{pip}$ sequence: 50 mM, 8 mM; same cell as in B.

the absence of a response to strophanthidin in the same cell with Na-free pipette solution.

Na/K pump I-V relationships obtained at various levels of $[Na]_{pip}$ in other cells exposed to Na-free external solution are illustrated in the upper panels of Fig. 7. These were all determined by subtracting I-V relationships obtained in 2 mM strophanthidin from control I-V relationships obtained in its absence, all at 5.4 mM $[K]_o$ and in the presence of 2 mM Ba, and thus should represent accurate estimates of Na/K pump current over the entire voltage range covered (Gadsby and Nakao, 1989). The most striking feature of these I-V curves is that they show pump current

to be only weakly voltage dependent in the absence of external Na, over a wide range of $[\text{Na}]_{\text{pip}}$, as already demonstrated in Figs. 1–3 for cells at 50 mM $[\text{Na}]_{\text{pip}}$ exposed to 1.5 mM $[\text{Na}]_{\text{o}}$. The lower graphs in Fig. 7 show normalized I-V relationships obtained by dividing pump current by its amplitude at +20 mV for each curve in the upper graphs. Within the error of the measurements, these normalized pump I-V relationships all seem to have the same relatively flat shape, which indicates that, in the absence of external Na, lowering $[\text{Na}]_{\text{pip}}$ from 50 mM to 3, 17, or 8 mM causes a reduction of Na/K pump current by about the same factor at all membrane potentials tested, in contrast to the effects at 150 mM $[\text{Na}]_{\text{o}}$ described above, where lowering $[\text{Na}]_{\text{pip}}$ clearly shifted as well as scaled down the pump I-V curves.

In Na-free external solution then, Na/K pump current shows relatively little voltage dependence, and activation of the pump by intracellular $[\text{Na}]$ seems independent of membrane potential.

Na/K Pump Current Activation by Intracellular $[\text{Na}]$

The results of varying intracellular $[\text{Na}]$ indicate that, both at 150 mM $[\text{Na}]_{\text{o}}$ and at zero $[\text{Na}]_{\text{o}}$, Na/K pump current amplitude is a saturable function of $[\text{Na}]_{\text{pip}}$. In analyzing that dependence on $[\text{Na}]_{\text{pip}}$, data obtained at 150 mM $[\text{Na}]_{\text{o}}$ were initially summarized separately from those at zero $[\text{Na}]_{\text{o}}$. Also, for convenience and to facilitate comparison with the large body of data already obtained on the Na/K pump of red blood cells, which have negligibly small membrane potentials (Hoffman and Laris, 1974), we focused our kinetic analysis on measurements at 0 mV. We made allowance for rundown as follows: for each cell, we normalized pump current amplitude at 0 mV, at a given test $[\text{Na}]_{\text{pip}}$, with respect to the average size at 0 mV of the pump current obtained in the two bracketing control runs at 50 mM $[\text{Na}]_{\text{pip}}$. It was not always possible to successfully complete the control run at 50 mM $[\text{Na}]_{\text{pip}}$ after each test $[\text{Na}]_{\text{pip}}$, but 60 of the 70 ratios summarized here for $[\text{Na}]_{\text{pip}} > 0$ mM were fully bracketed in that way. A nonlinear least-squares fit of the Hill equation to the results obtained from 25 cells exposed to Na-free external solution yielded a $K_{0.5}$ of 10.0 ± 0.7 mM and a Hill coefficient (n_{H}) of 1.37 ± 0.11 , whereas a similar fit to the data from eight cells studied at 150 mM $[\text{Na}]_{\text{o}}$ gave $K_{0.5} = 10.9 \pm 0.9$ mM and $n_{\text{H}} = 1.40 \pm 0.14$. Because the results shown above in Fig. 3 indicate that, at 0 mV (and at 50 mM $[\text{Na}]_{\text{pip}}$), pump current amplitude at 150 mM $[\text{Na}]_{\text{o}}$ is within 5% of that in Na-free external solution, the data obtained under these two conditions have been combined in the graph of Fig. 8. The smooth curve drawn through the points shows the best fit of the Hill equation to the combined data from all 33 cells, which gave $K_{0.5} = 10.0 \pm 0.5$ mM, $n_{\text{H}} = 1.36 \pm 0.07$, and a maximal pump current at saturating $[\text{Na}]_{\text{pip}}$ of 1.13 times that at 50 mM $[\text{Na}]_{\text{pip}}$.

As already mentioned, the rightward shift of the normalized pump I-V relationships seen on lowering $[\text{Na}]_{\text{pip}}$ at 150 mM $[\text{Na}]_{\text{o}}$ (Figs. 4 and 5) implies a greater sensitivity of the pump to intracellular $[\text{Na}]$ at more positive potentials. Further analysis of the four most complete experiments (including the one represented in Fig. 4) from the eight carried out at 150 mM $[\text{Na}]_{\text{o}}$, revealed that at -80 , -60 , and 0 mV, respectively, the $K_{0.5}$ for $[\text{Na}]_{\text{pip}}$ was 10.7 ± 1.1 mM, 9.5 ± 0.7 mM, and 7.8 ± 0.7 mM, and the corresponding n_{H} was 2.2 ± 0.5 , 2.4 ± 0.4 , and 1.6 ± 0.2 . Although the data are few and scattered, their trend over this limited voltage range

suggests an e -fold drop in the $K_{0.5}$ for about a 250-mV positive shift of membrane potential.

Influence of Extracellular [K] at High [Na]_o

The effect on the steady-state Na/K pump I-V relationship of varying $[K]_o$ between 0 and 10 mM was examined in cells equilibrated with 50 mM Na, 10 mM ATP pipette solution, by substituting K for Na (or vice versa) in the external solution. Fig. 9 shows some results from one of these experiments. The chart recording (Fig. 9 A) reveals that, at -40 mV, strophanthidin-sensitive current was negligibly small in K-

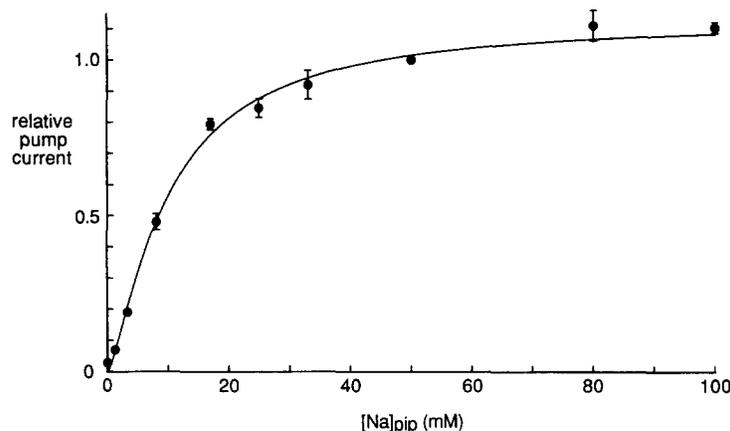


FIGURE 8. Saturable activation of Na/K pump current at 0 mV by $[Na]_{pip}$. For each level of $[Na]_{pip}$, pump current amplitude at 0 mV was normalized to the average of its sizes in the two bracketing runs at 50 mM $[Na]_{pip}$. Mean values (\pm SEM) of the resulting relative pump currents, obtained from 33 cells, are plotted against $[Na]_{pip}$: for eight cells, $[Na]_o$ was 150 mM and the strophanthidin concentration was 0.5–2 mM; for five cells, $[Na]_o$ was zero and the strophanthidin concentration was 2 mM; for the remaining 20 cells, $[Na]_o$ was zero and 0.5 mM strophanthidin was applied in K-free solution. The curve shows the least-squares fit to the combined data (unweighted), from all 33 cells, of the Hill equation: relative current = (maximal relative current)/ $\{1 + (K_{0.5}/[Na]_{pip})^{n_H}\}$, where maximal relative current = 1.13 ± 0.02 , $K_{0.5} = 10.0 \pm 0.5$ mM, and $n_H = 1.36 \pm 0.07$. During pumping, $[K]_o$ was 5.4 mM for all cells; four of the eight cells studied at 150 mM $[Na]_o$ were also exposed to 0.1 mM $LaCl_3$ to reduce leakage currents.

free solution but large (~ 200 pA) at 5.4 mM $[K]_o$; it was almost as large at 2.7 mM $[K]_o$, but only half that size at 1 mM $[K]_o$. It is noteworthy that in the presence of strophanthidin the holding current was the same at all $[K]_o$ levels, confirming that under the conditions of these experiments, at least at -40 mV, all $[K]_o$ -sensitive current other than Na/K pump current had been abolished (cf. Gadsby et al., 1985; Gadsby and Nakao, 1989). The strophanthidin-sensitive I-V relationships from this experiment are presented in Fig. 9 B. They show the usual, sigmoid voltage dependence of pump current at the higher K concentrations, and suggest that this voltage dependence is retained even when the pump rate is halved by lowering $[K]_o$ to 1 mM.

Because of the scatter of the measurements, it is not clear from Fig. 9 *B* whether lowering $[K]_o$ simply scaled down pump current by a constant factor at all membrane potentials. Fig. 9 *C* shows the same pump I-V relationships, for 1, 2.7, and 5.4 mM $[K]_o$, after normalizing each curve to its amplitude at +20 mV. Here also, the answer is not clear-cut but the results suggest that lowering $[K]_o$ from 5.4 to 2.7 mM more or less simply scaled down pump current (by ~10% in this case), whereas lowering $[K]_o$ to 1 mM further reduced pump current to ~50% and seemed to shift the

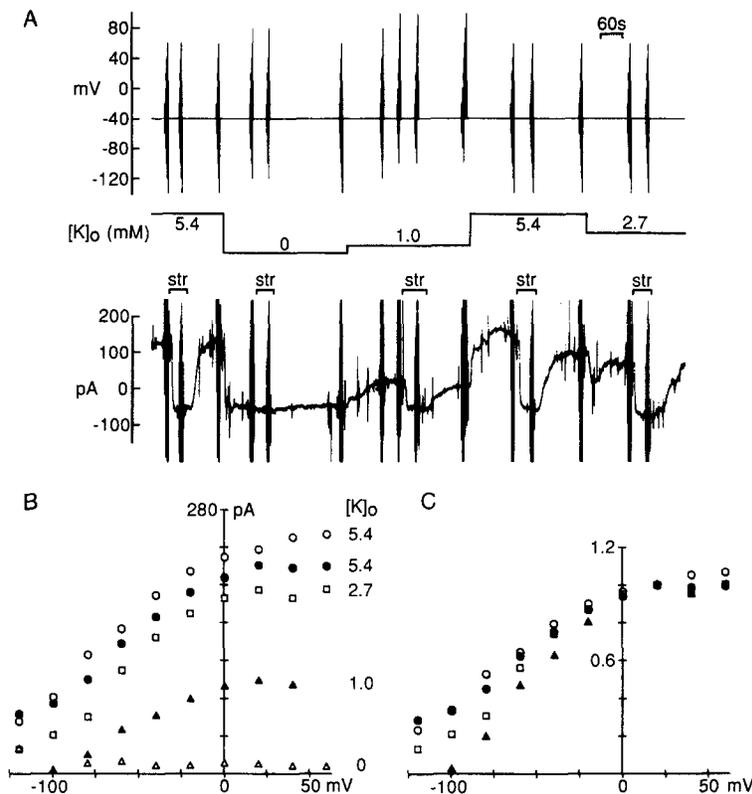


FIGURE 9. Influence of $[K]_o$ on strophanthidin-sensitive whole-cell currents at high $[Na]_o$. (A) Chart records of voltage and current, the middle line indicating changes of $[K]_o$, and the bars (*str*) marking exposures to 0.5 mM strophanthidin. (B) Steady-state Na/K pump I-V relationships from A, at the $[K]_o$ levels indicated, obtained by subtraction of I-V data as in Fig. 1. (C) Same data and symbols as in B (but 0 mM $[K]_o$ data omitted), except each I-V curve normalized to its amplitude at +20 mV. $[Na]_{pip}$, 50 mM; $[Na]_o$, ~150–155 mM; cell capacitance, 218 pF; initial pipette resistance, 1.3 M Ω .

I-V relationship (by ~25 mV) toward more positive potentials. This rightward shift was a consistent finding and in the seven cells that yielded full I-V curves at 1 mM $[K]_o$ its average size (\pm SD), at a normalized pump current amplitude of 0.5, was 40 ± 7 mV (five of these seven values were bracketed). The switch from 5.4 to 2.7 mM $[K]_o$, however, elicited a barely discernible rightward shift of the normalized

pump I-V curve which averaged 15 ± 6 mV in three bracketed experiments. Again, these shifts occurred in the same direction as, but were smaller than, the expected shifts of $E_{Na/K}$ which, on lowering $[K]_o$ from 5.4 mM to 2.7 or 1 mM, should be +37 and +90 mV, respectively.

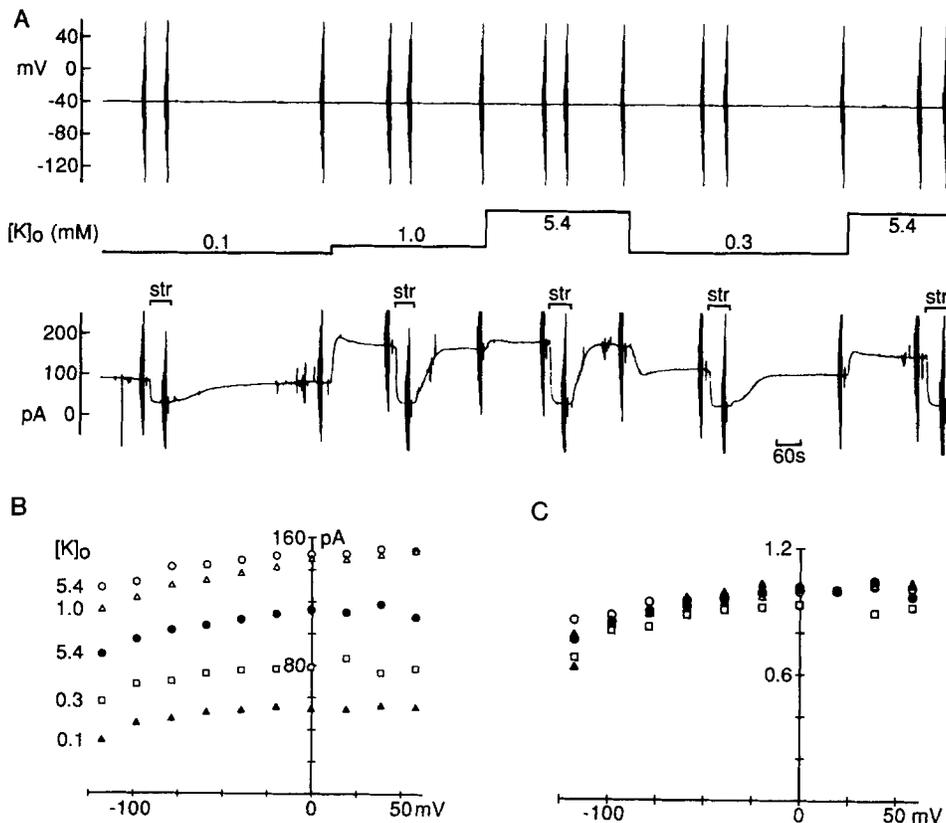


FIGURE 10. Influence of $[K]_o$ on strophanthidin-sensitive whole-cell currents at zero $[Na]_o$. (A) Chart records of voltage and current, the middle line indicating changes of $[K]_o$ (note the low effective concentration range), and the bars (*str*) marking exposure to 2 mM strophanthidin (note that strophanthidin wash-off appears slower at lower $[K]_o$; see also Fig. 9). (B) Steady-state Na/K pump I-V relationships from A, at the $[K]_o$ levels indicated, obtained by subtraction of I-V data collected in the presence of strophanthidin from that determined after washing it off (●, □, ▲), or from the average of the I-V curves determined in its absence, both before and after (○, △). (C) Same data and symbols as in B, but each I-V curve normalized to its amplitude at +20 mV. $[Na]_{pip}$, 50 mM; $[Na]_o$, 0 mM; cell capacitance, 149 pF; initial pipette resistance, 0.9 MΩ.

Influence of Extracellular [K] at Zero [Na]_o

It is well known from experiments on red blood cells (Post et al., 1960; Garrahan and Glynn, 1967) and squid axons (Baker et al., 1969; Rakowski et al., 1989) that the apparent affinity of the pump for external K is greatly enhanced when $[Na]_o$ is lowered. The results in Fig. 10, obtained in Na-free, NMG-containing external solu-

tion, demonstrate the same effect in an isolated cardiac myocyte. The chart recording in Fig. 10 A confirms once more that, in the presence of strophanthidin, the holding current was insensitive to changes in $[K]_o$, so that $[K]_o$ -induced current shifts in the absence of strophanthidin reflect changes in pump current. It is evident that, at -40 mV, pump current was almost as large at 1 mM $[K]_o$ as it was at 5.4 mM, was more than half that size at 0.3 mM $[K]_o$, and was substantial even at 0.1 mM $[K]_o$.

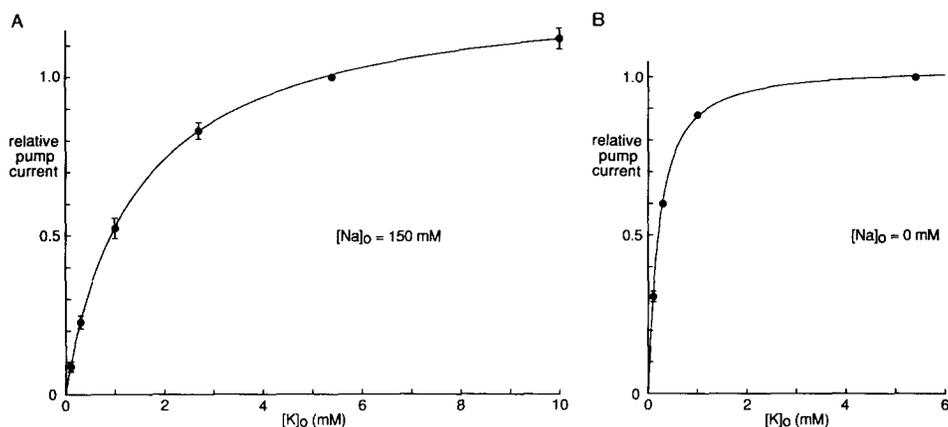


FIGURE 11. Activation of Na/K pump current at 0 mV by $[K]_o$, at high $[Na]_o$ (A) or at zero $[Na]_o$ (B). In both cases, pump current amplitude at 0 mV at each $[K]_o$ was normalized to its average size in the two bracketing runs at 5.4 mM $[K]_o$ (or to its size in the sole adjacent run at 5.4 mM $[K]_o$, if necessary). The graphs show mean values (\pm SEM) of the resulting relative pump currents plotted against $[K]_o$, and the curves show least-squares fits of the Hill equation to the unweighted data (as in Fig. 8). $[Na]_{pp}$ was 50 mM in all cells. (A) Data from 17 cells at high $[Na]_o$; best fit parameters: maximal relative current = 1.30 ± 0.10 , $K_{0.5} = 1.50 \pm 0.32$, $n_H = 0.96 \pm 0.13$. Results obtained at 0 mM $[K]_o$ are omitted; relative pump current at 0 mM $[K]_o$ averaged 0.054 ± 0.007 in 18 measurements on 15 cells, but including these data did not alter the derived fit parameters (maximal relative current = 1.32 ± 0.10 ; $K_{0.5} = 1.54 \pm 0.31$; $n_H = 0.95 \pm 0.11$). A contamination of the nominally K-free fluid by ~ 80 μ M K would be required to account for that current by Na/K exchange, but atomic absorption spectrometry revealed only 31 μ M K. Most of the 0 $[K]_o$ response must therefore be attributed either to remaining systematic error or to electrogenic Na/Na exchange (e.g., Lee and Blostein, 1980). (B) Data from four cells at zero $[Na]_o$; no data collected in 0 mM $[K]_o$ fluid which, however, was shown to contain ≤ 2 μ M K; best fit Hill equation parameters: maximal relative current = 1.03 ± 0.05 , $K_{0.5} = 0.22 \pm 0.03$ mM, $n_H = 1.12 \pm 0.14$. (The important result, the marked increase in the apparent affinity for external K on removal of external Na, is clearly independent of this fit of four mean values with three parameters.)

These observations are corroborated by the corresponding pump I-V relationships in Fig. 10 B. As in all other experiments carried out in the absence of external Na, pump current was only weakly voltage dependent, and lowering $[K]_o$ seemed to simply scale down the relatively flat pump I-V relationships. This is demonstrated by the normalized pump I-V relationships in Fig. 10 C which are all approximately superimposed, despite some variability at extreme positive and negative potentials.

Na/K Pump Current Activation by Extracellular [K]

The graphs in Fig. 11 summarize our results on the activation of Na/K pump current by $[K]_o$ either in the presence of high $[Na]_o$ (~145–155 mM; Fig. 11 A), or in Na-free solutions (Fig. 11 B). The analysis was limited to pump currents measured at 0 mV. Whenever possible, for each level of $[K]_o$, relative pump current was estimated by normalizing the pump current obtained at that test $[K]_o$ to the average size of the pump currents at 5.4 mM $[K]_o$, determined before and after the test. To facilitate data collection, two different concentrations were sometimes tested between the two control measurements (e.g., Figs. 9 and 10) but, even so, fully bracketed current ratios could not always be obtained. Of the 39 ratios included in the two graphs of Fig. 11, only 24 were fully bracketed.

The data obtained from 17 cells at high $[Na]_o$ (Fig. 11 A) were well approximated by the Hill equation (smooth curve) with a $K_{0.5}$ of 1.5 ± 0.3 mM, a n_H of 1.0 ± 0.1 , and a maximal pump current at saturating $[K]_o$ 1.3 times that at 5.4 mM $[K]_o$. The fewer results obtained from four cells in Na-free fluid were also well described by the Hill equation (Fig. 11 B) but in that case the apparent $K_{0.5}$ was 0.22 ± 0.03 mM, the n_H was 1.1 ± 0.1 , and the estimated maximal pump current was only 1.03 times that at 5.4 mM $[K]_o$.

DISCUSSION

We have determined steady-state I-V relationships for the forward-running Na/K pump over a relatively wide range of ionic conditions. The results demonstrate that, at a fixed membrane potential, outward pump current is a saturable function of the concentration of either transported ion, intracellular Na or extracellular K, and that the resulting activation curves are only weakly influenced by membrane potential. At a normal physiological level of $[Na]_o$, outward pump current is strongly voltage dependent over the full range of pump activation by $[Na]_{pip}$ and by $[K]_o$. Extracellular $[Na]$, however, exerts a major influence on the voltage dependence of pump current.

Possible Sources of Error

The two main sources of uncertainty in these experiments are the variable, slow rundown of pump current, and the limited control over the concentrations of Na and K ions at the internal and external faces of the cell membrane, respectively, when the pump rate is high.

As reported in the preceding paper (Gadsby and Nakao, 1989), the rundown occurred with a roughly exponential time course, on average, reducing pump current to about half its initial amplitude without altering the shape of the pump I-V relationship. Approximate allowance for the effects of rundown was made, as far as possible, by bracketing test measurements between two control runs, and then using linear interpolation to obtain appropriate control values for comparison with the test results. Unbracketed (single-sided) comparisons usually resulted from deterioration of the preparation before completion of the second control run and so would have contributed a tendency towards underestimation of the normalized pump current for test ion concentrations both smaller and larger than the control. Because

the control concentrations, 50 mM $[\text{Na}]_{\text{pip}}$ and 5.4 mM $[\text{K}]_o$, were both relatively close to saturating (88 and 77%, respectively), our inclusion of some unbracketed ratios must have led to, if anything, overestimation of the $K_{0.5}$ for pump activation by $[\text{Na}]_{\text{pip}}$ and by $[\text{K}]_o$.

During steady-state forward cycling of the Na/K pump reaction, concentration gradients of the transported ions, Na and K, will exist both inside and outside the cell due to restricted diffusion between the cell interior and the pipette, and between the cell surface and the bathing fluid. K accumulation and Na depletion (with respect to pipette concentrations) are expected inside the cell, and K depletion and Na accumulation (with respect to bath concentration) are expected outside the cell.

Extracellular concentration gradients should not have been large. For a 3Na/2K pump transport stoichiometry, extracellular Na accumulation should be ~1.5 times the depletion of K, if the diffusion barrier is nonselective. Because pump current was absent at zero $[\text{K}]_o$, the magnitude of K depletion at the cell surface must have been <5.4 mM when $[\text{K}]_o$ was 5.4 mM. External Na accumulation should therefore have been insignificant when $[\text{Na}]_o$ was 150 mM, and should have had little effect even at 0 mM $[\text{Na}]_o$, because external Na seems to act with low affinity (cf. Fig. 3). With regard to external K, pump-induced depletion should have influenced only the Na/K pump itself, because changes in $[\text{K}]_o$ between zero and 5.4 mM (in the presence of 2 mM Ba) had no effect on any component of membrane current other than that generated by the Na/K pump (see Fig. 5 C of Gadsby and Nakao, 1989). Whereas the size of the effect is unknown, its direction is clear: the $K_{0.5}$ values for $[\text{K}]_o$, 1.5 mM at 150 mM $[\text{Na}]_o$ and 0.2 mM in Na-free solution, must be overestimates.

Intracellular concentration gradients could have been substantial. Following reduction of $[\text{Na}]_{\text{pip}}$ to 3 mM, the final approach to the new steady current level (e.g., Figs. 4–6) occurred with a half-time of some 15–30 s, or a time constant of roughly 20–45 s, presumably the time constant (τ_{pip}) for diffusional equilibration across the resistance at the pipette tip (Pusch and Neher, 1988; Oliva et al., 1988), because pump current is nearly proportional to $[\text{Na}]_{\text{pip}}$ over that range (Fig. 8) and equilibration within the cell is expected to be much faster. In the steady state, the pumped efflux of Na (Φ_{Na} mol·s⁻¹) from the cell should equal the net diffusive flux of Na from pipette to cell, insofar as electrodiffusive leak of Na from bath to cell can be ignored (see below). Thus,

$$\Phi_{\text{Na}}/v = ([\text{Na}]_{\text{pip}} - [\text{Na}]_i)/\tau_{\text{pip}}, \quad (1)$$

where v (cm³) represents the cell volume accessible to Na ions, and $[\text{Na}]_i$ is the intracellular Na concentration. For a 3Na/2K transport ratio, $\Phi_{\text{Na}} = 3I_p/F$, where I_p (A) is whole-cell pump current and F (10⁵ C·mol⁻¹) is Faraday's constant. Because under conditions of strong pump activation by, say, 50 mM $[\text{Na}]_{\text{pip}}$, pump current was ~150 pA at the holding potential, -40 mV, and cell volume was likely on the order of 20 pl (2×10^{-8} cm³; e.g., for our average cell dimensions of 126 $\mu\text{m} \times 31 \mu\text{m} \times \sim 5 \mu\text{m}$; see Gadsby and Nakao, 1989; cf. Imanaga et al., 1987), the concentration gradient, $[\text{Na}]_{\text{pip}} - [\text{Na}]_i$, is estimated to be ~5–10 mM at 50 mM $[\text{Na}]_{\text{pip}}$. If transmembrane Na influx (leak) were substantial and/or if the cell volume

were larger (cf. Silver and Houser, 1985), then the estimated gradient would be smaller. In any event, the following argument suggests that the gradient must be <20 mM at 50 mM $[\text{Na}]_{\text{pip}}$: if it were 20 mM, then because $[\text{Na}]_{\text{pip}} - [\text{Na}]_i$ scales with Φ_{Na} (Eq. 1), the gradient would have to be 10 mM when Φ_{Na} is halved, i.e., at ~ 10 mM $[\text{Na}]_{\text{pip}}$ (Fig. 8), so that $[\text{Na}]_i$ would then have to be zero, which is clearly impossible.

Some leak of Na ions from the bath into the cell is suggested by our observation that when $[\text{Na}]_{\text{pip}}$ was zero, relative pump current (Fig. 8) averaged $3.5 \pm 2.1\%$ (\pm SD) in seven cells at 150 mM $[\text{Na}]_o$, but only $0.8 \pm 0.5\%$ in six other cells bathed in Na-free solution. But, inspection of Fig. 8 suggests that intracellular $[\text{Na}]$ could not have been more than ~ 2 mM at 0 mM $[\text{Na}]_{\text{pip}}$ and 150 mM $[\text{Na}]_o$. Cellular Na accumulation of that order is consistent with Fig. 1 A, in which lowering $[\text{Na}]_o$ from 150 to 1.5 mM, in the presence of strophanthidin, resulted in a 60-pA outward shift of holding current presumably reflecting loss of inward Na leak. If passive Na influx were -60 pA, and pumped Na efflux negligible, in a typical cell equilibrated with 0 mM $[\text{Na}]_{\text{pip}}$ then, with τ_{pip} and v as above, $[\text{Na}]_i$ is calculated to be 0.6–1.4 mM.

Pump-mediated intracellular K accumulation is expected to have been one-third smaller than the concomitant depletion of Na and so should have had little influence on the pump rate (cf. Garay and Garrahan, 1973). We conclude that, because some depletion of intracellular Na probably occurred at high Na/K pump rates, the $K_{0.5}$ estimated by varying $[\text{Na}]_{\text{pip}}$ must be an upper limit for pump activation by $[\text{Na}]_i$.

Na/K Pump Activation by Intracellular Na at 0 mV

Despite these sources of error, our extracted data for activation of outward pump current by $[\text{Na}]_{\text{pip}}$ at 0 mV ($K_{0.5} = 10$ mM, $n_H = 1.4$) compare well with previous results on $[\text{Na}]$ dependence of pump fluxes in human red blood cells (e.g., Sachs, 1970, 1977, 1986; Garay and Garrahan, 1973), which have extremely small membrane potentials little affected by changes in pump rate (Hoffman and Laris, 1974), and with similar results on pump current across the basolateral membranes of turtle colon (Halm and Dawson, 1983) or rabbit urinary bladder (Eaton et al., 1982) both voltage clamped at 0 mV: all yielded reasonable fits to the Hill equation with $K_{0.5}$ values of 8–17 mM and n_H values of 1.3–1.6 (reviewed in Karlisch and Stein, 1985). Working with reconstituted Na/K pumps in phospholipid vesicles, Karlisch and Stein (1985) analyzed extensive data on activation of ATP-dependent Na/K exchange by cytoplasmic $[\text{Na}]$ (when cytoplasmic $[\text{K}]$ was zero) in terms of the Hill equation, and found $n_H = 1.9$, $K_{0.5} = 7$ mM at 0 mM $[\text{Na}]_o$, and $n_H = 1.3$, $K_{0.5} = 10$ mM at 100 mM $[\text{Na}]_o$. Given the uncertainty of the true cytoplasmic $[\text{Na}]$ during pumping in our experiments, it is perhaps not surprising that the $K_{0.5}$ and n_H derived from Hill fits to our data at 0 mM $[\text{Na}]_o$ (10 ± 1 mM and 1.4 ± 0.1) and at 150 mM $[\text{Na}]_o$ (11 ± 1 mM and 1.4 ± 0.1) were indistinguishable.

The approximately linear relationship between $[\text{Na}]_i$ and pump rate, estimated from net loss of Na or from pump current, found in earlier studies in cardiac tissue (reviewed in Gadsby, 1984) most likely reflects the limited range of $[\text{Na}]_i$ examined, usually within a few millimolar of its normal resting level of 5–10 mM; over that range, pump rate is indeed roughly proportional to $[\text{Na}]_i$ (Fig. 8). Subsequent inves-

tigations over a more extensive range have yielded saturating activation curves. In cardiac sarcolemmal vesicles, ATP hydrolysis and ATP-dependent Na influx via inside-out Na/K pumps were both activated by cytoplasmic [Na] along the same sigmoidal curve, which gave $K_{0.5} \approx 9$ mM and $n_H = 2.8$ (Philipson and Nishimoto, 1983); cytoplasmic [K] was only 25 mM and there was no Na on the opposite side of the membrane. Recently, Sejersted et al. (1988) found that net Na loss from Na-loaded sheep Purkinje fibers, monitored with Na-sensitive microelectrodes, was a sigmoidal function of intracellular Na activity, with average $K_{0.5} = 14$ mM and $n_H = 1.9$. Surprisingly, however, these authors reported that the maximal rate of Na loss, measured at a membrane potential near -70 mV, was independent of $[Na]_o$, in contrast to the substantial activation of the pump seen on lowering $[Na]_o$ at such negative membrane potentials in myocytes (Figs. 1–3) and in squid giant axon (Rakowski et al., 1989).

Na/K Pump Activation by Extracellular K at 0 mV

Our data for K_o activation at 150 mM $[Na]_o$ are consistent with previous results from heart (reviewed in Gadsby, 1984; cf., Falk and Cohen, 1984; Cohen et al., 1987). The upper estimate of 1.5 mM for the $K_{0.5}$ at 150 mM $[Na]_o$ also compares favorably with values (1.3–2.1 mM) derived from transport data on red cells (Post et al., 1960; Garrahan and Glynn, 1967), as does the marked increase in apparent affinity for external K on withdrawal of extracellular Na, under which condition Post et al. (1960) and Garrahan and Glynn (1967) found the $K_{0.5}$ to be 0.4 mM and 0.14–0.19 mM, respectively, in comparison with our figure of 0.22 mM. That enhanced K affinity on Na removal probably accounts for difficulty in arresting the pump by means of K-free bathing fluid at very low $[Na]_o$, especially in multicellular cardiac preparations (e.g., Achenbach, 1988) in which restricted extracellular diffusion maintains a nonzero [K] at the cell surface.

Voltage Dependence of Pump Activation by Intracellular Na and by Extracellular K

At 150 mM $[Na]_o$, the shape of the pump I-V relationship seemed little altered when the pump rate was changed by varying either $[Na]_{pip}$ (Figs. 4 and 5) or $[K]_o$ (Fig. 9). This result contrasts with recently reported data both from *Xenopus* oocytes (Eisner et al., 1987), which suggested that pump current was voltage sensitive in Na-loaded (~ 30 mM) oocytes, but voltage insensitive at normal low intracellular [Na] (~ 8 mM), and with recent results from sheep cardiac Purkinje myocytes exposed to 100 mM $[Na]_o$ (Glitsch et al., 1989) which suggest the opposite, that pump current is voltage sensitive at low, but much less so at high, internal [Na]. While we can offer no explanation for the *Xenopus* results, other than the difficulty of reliably extracting a small signal, the Purkinje myocyte results might be partly explained by the rightward shift of the pump I-V curve at low internal [Na], shown in Figs. 4 and 5, together with a diminished voltage sensitivity of pump current due to the somewhat reduced $[Na]_o$ of 100 mM (cf. Fig. 3, above).

That rightward shift of the normalized pump I-V relationship on lowering $[Na]_{pip}$ (Figs. 4 and 5) implies that, at 150 mM $[Na]_o$, the apparent affinity of the pump for cytoplasmic Na is greater at positive than at negative potentials. Our limited results suggest that the $K_{0.5}$ would be reduced e -fold by a roughly 250-mV positive shift of

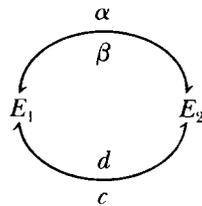
membrane potential. Goldshlegger et al. (1987) recently reported a similar decline of the $K_{0.5}$ for cytoplasmic Na activation of reconstituted pumps in vesicles as the membrane potential was made more positive. They interpreted this effect in terms of an influence of voltage on Na binding, possibly indicating that one of the pump's three binding sites for cytoplasmic Na ions resides in a shallow ion well (Mitchell, 1969; Läuger and Apell, 1986) which experiences about one-tenth of the membrane field. However, in the present experiments, when $[Na]_o$ was zero the rather flat pump I-V relationships seemed to be scaled in a roughly voltage-independent manner when $[Na]_{pip}$ was varied (Fig. 7), suggesting that pump activation by intracellular Na (and, hence, binding of cytoplasmic Na) was then also independent of membrane potential. Furthermore, the main effect of changes in $[Na]_{pip}$ appears to be alteration of the saturating level of pump current at large positive potentials (i.e., an effect on the voltage-insensitive, rate-limiting reaction constant; see below), and this effect is the same irrespective of the presence or absence of extracellular Na (Figs. 4–8). Thus, barring a *trans* effect of external Na (Karlsh and Stein, 1985) causing an allosteric disturbance sufficient to render cytoplasmic Na binding voltage sensitive, an influence of membrane potential on cytoplasmic Na ion binding seems unlikely. The weak voltage sensitivity of the $K_{0.5}$ for $[Na]_{pip}$ at 150 mM $[Na]_o$ described here might reflect, instead, voltage-dependent redistribution of enzyme intermediates in the reaction cycle (Hansen et al., 1981; Chapman et al., 1983). It is puzzling that the vesicle experiments of Goldshlegger et al. (1987) which demonstrated measurable voltage dependence were carried out in the nominal absence of extracellular Na, where we find little evidence for any voltage dependence (Fig. 7); this apparent discrepancy remains to be explained.

Similar arguments apply in the case of pump activation by external K ions. As described in connection with Fig. 9, the normalized pump I-V relationship at 150 mM $[Na]_o$ was shifted rightwards on lowering $[K]_o$, implying that the $K_{0.5}$ for pump activation by $[K]_o$ (like that for activation by $[Na]_{pip}$) declines, or the apparent affinity increases, as the membrane potential is made more positive. This change, however, occurs in the direction opposite to that expected if extracellular K ions had to traverse part of the membrane field before binding to the Na/K pump. Just as for changes in $[Na]_{pip}$, the major influence of varying $[K]_o$ is on the saturating level of pump current at large positive potentials, both in the presence (Fig. 9) and in the absence (Fig. 10) of external Na. Once again, the apparent voltage-independent scaling of the pump I-V relationships in Na-free solution, this time by changes in $[K]_o$ (Fig. 10), implies that K binding to sites on the pump accessible from the extracellular surface is insensitive to membrane potential. Clearly, in the case of K_o activation, the voltage dependence of the $K_{0.5}$ at 150 mM $[Na]_o$ must reflect cycle properties other than K ion binding.

Shape of the Pump I-V Relationship and Influence of $[Na]_o$

As previously discussed (De Weer et al., 1988; Bahinski et al., 1988; Gadsby and Nakao, 1989), there is no compelling evidence that the Na/K pump reaction cycle includes more than a single electrogenic step (Karlsh et al., 1985; Nakao and Gadsby, 1986; Goldshlegger et al., 1987; Bahinski et al., 1988), which occurs late in Na translocation and could reflect deocclusion of Na ions or their subsequent

release (Borlinghaus et al., 1987; Apell et al., 1987). Because none of the alterations of [Na] or [K] reported here introduced a negative slope region into the Na/K pump I-V relation, despite large changes in overall driving force for the pump reaction cycle, there is no reason to modify this view. The following two-state scheme (Hansen et al., 1981),



can thus be considered a valid reduction of the customary Post-Albers scheme for the Na/K pump reaction cycle under a fixed set of ionic and metabolic conditions. Suitable values for the voltage-sensitive (α , β) and voltage-insensitive (c , d) pseudo first-order rate constants can easily be found to permit faithful description of the steady-state I-V relationship for the strongly activated Na/K pump (Gadsby and Nakao, 1989). (It is important to bear in mind that the empirical rate constants, α , β , c , d , of the pseudo two-state cycle, obtained by analyzing pump I-V relationships, are related to the real rate constants of the real multistate cycle by so-called "reserve factors" [Hansen et al., 1981], which are functions of all the real voltage-insensitive rate constants and which account for enzyme occupied in the voltage-insensitive pathway). As already mentioned, varying the concentrations of the transported ions influences pump current amplitude in a more or less voltage-independent manner, consistent with modification of a relatively slow voltage-insensitive step, represented here by c (Hansen et al., 1981).

The influence of external [Na], however, is complex. Although the voltage dependence of pump current is consistently diminished on switching to Na-free fluid, the current amplitude at 0 mV seems enhanced by ~20% in squid axon (Rakowski et al., 1989), or by ~5% in myocytes (Figs. 1 and 3, above), but is substantially reduced in *Xenopus* oocytes (Béhé and Turin, 1984; 1984; Rakowski and Paxson, 1988). An increase of some 20–30%, due to removal of competitive Na binding, might be expected in both squid axon and myocyte on the basis of their K_o activation curves, determined in parallel experiments (e.g., Fig. 11, above). In the absence of appropriate kinetic data, the reduced current in oocytes (which were not internally dialyzed) is probably attributable to a fall of [Na]_i; the shortfall in myocytes is more difficult to account for, but might also reflect a small drop in the [Na] just beneath the cell surface when [Na]_o is lowered towards zero.

These effects at 0 mV notwithstanding, the striking influence of [Na]_o on the shape of the pump I-V curve might seem to imply, as already mentioned, a direct modulation of the charge-translocating step. But Hansen et al. (1981) have pointed out that manipulation of a fast, energetically downhill release step that is voltage insensitive but immediately follows the charge transit step can cause an apparent change in α , the forward voltage-sensitive rate constant, via alteration of the distribution of enzyme intermediates (reserve factors). Thus, if Na deocclusion were voltage sensitive, and Na release to the exterior voltage-insensitive but fast then lower-

ing $[Na]_o$ would result in a proportional apparent speeding of forward charge transit. In that case, however, the voltage-insensitive forward rate constant, c , should be increased by the same factor (Hansen et al., 1981) and so pump current at positive potentials should be greatly enhanced at low $[Na]_o$. No such large amplification was seen in our experiments, even at zero $[Na]_o$. An apparent effect of external Na ions therefore seems much less likely than a direct influence of $[Na]_o$ on charge translocation.

The influence would be direct if the pseudo first-order rate constant, β , contained $[Na]_o$. Our preliminary observations on the $[Na]_o$ dependence of the transient pump currents elicited by voltage jumps in K-free fluids (Nakao and Gadsby, 1986) indicate that raising $[Na]_o$ does, indeed, increase their decay rate constants at large negative potentials (Nakao, M., and D.C. Gadsby, unpublished observation). If, in addition, an asymmetric voltage dependence were to render β severalfold more sensitive to changes of voltage than α (Nakao and Gadsby, 1986), i.e., if the energy barrier to charge movement is assumed to lie close to the cytoplasmic boundary of the membrane field (e.g., Läuger and Apell, 1986), then the effect of removing external Na could be explained easily: in the absence of external Na, pump current would display the weak voltage sensitivity of α , whereas at high $[Na]_o$ an increasing backflux through the charge translocation step on hyperpolarization would cause a progressive decline of net outward pump current (Fig. 3, above; cf. Eisner et al., 1987). However, that kind of explanation has been constrained by the recent demonstration in the squid axon that unidirectional ^{22}Na efflux and pump current have the same voltage dependence, which is steeper at high $[Na]_o$ than with Na-free external solution (Rakowski et al., 1989). This equivalence of unidirectional Na efflux and net flux means that unidirectional Na influx must be zero which, in turn, means that there must be a practically irreversible step between intracellular Na binding and Na translocation (cf. De Weer, 1990). The voltage-insensitive limb of the two-state scheme represents just such an irreversible step, because we have argued above (a) that the effects on pump I-V curves of altering $[Na]_{pip}$ are consistent with changes in the forward voltage-insensitive rate constant, c , and (b) that the large negative reversal potential implied by our pump I-V curves (Gadsby and Nakao, 1989) suggests that the backward voltage-insensitive rate constant, d , is extremely small. The computed I-V curves in Fig. 3 confirm that, by allowing β^0 to vary, while α^0 (490 s^{-1}), c (61 s^{-1}), d (0 s^{-1}), and δ (0.1) are held constant at the values given by a nonlinear least-squares fit to the data obtained at 1.5 mM $[Na]_o$ (for which β^0 was set to zero), this simple scheme can mimic the results of experiments in which $[Na]_o$ was varied (Figs. 1–3).

The low apparent affinity for this effect of $[Na]_o$ on turnover rate near 0 mV (Fig. 3) might account for the lack of influence of 100 mM $[Na]_o$ on ^{22}Na efflux during the first turnover of Na/K pumps in vesicles after flash photolysis of caged ATP (Forbush, 1984). The enhanced apparent affinity at large negative potentials could conceivably be explained by redistribution of enzyme intermediates, but is also consistent with an extracellular Na ion (or, possibly, ions) having to bind at a site deep within the membrane field to affect outward pump current (e.g., Läuger and Apell, 1988). There is no evidence yet, however, that such an ion well lies in the Na transport path. If there were a well, and if K import used the same route, then the apparent affinity for extracellular K ions should also increase on hyperpolarization. But,

as already discussed, at 150 mM $[Na]_o$ the apparent K affinity was slightly decreased on hyperpolarization and, anyway, the simple voltage-independent scaling of the pump I-V relationship by changes in $[K]_o$, at zero $[Na]_o$, argues against any substantial ion well in the (extracellular) K translocation path.

The well-known competitive interaction between extracellular K and Na ions presumably does occur in the transport path, but there is no evidence for any voltage dependence. Indeed, the results presented here are consistent with two effects of external Na ions, one mediated by changes in the voltage-sensitive rate constant, β , and the second apparently modifying (just as changes in $[K]_o$ appear to do) the voltage-insensitive rate constant, c . Thus Fig. 1 shows that lowering $[Na]_o$ in the presence of near saturating $[K]_o$ simply diminishes the voltage dependence of pump current by augmenting it at negative potentials, without much affecting the voltage-independent current level at positive potentials, suggestive of a decrease in β . In contrast, Figs. 9–11 indicate that withdrawal of external Na in the presence of only 1 mM $[K]_o$ (approximately half-saturating at 150 mM $[Na]_o$, but close to saturating at 0 mM $[Na]_o$) does not merely diminish the voltage dependence of pump current at negative voltages, but simultaneously almost doubles the voltage-independent current at positive voltages, suggestive of both a decrease in β and an increase in c . It was recently suggested (Jørgensen and Andersen, 1988) that Na translocation might include a conformational transition, $E_1(Na_3)-P \rightarrow E_2(Na_2)-P$, in which one of the three occluded Na ions is released to the outside before deocclusion of the remaining two Na ions. If that novel conformational change were the sole voltage-sensitive transition, then the Na/K transport cycle would include two steps that could be influenced by changes of $[Na]_o$: a voltage-independent competitor with external K ions (evident as an effect on c), and a voltage-dependent interaction with occluded-Na states (possibly evident as an effect on β).

Apparently, further progress in analyzing Na/K pump I-V relationships will require a clearer understanding of the charge translocating step and of its modulation by the extracellular Na ion concentration. Detailed examination of the $[Na]_o$ dependence both of transient pump currents in the absence of external K (Nakao and Gadsby, 1986) and of steady-state pump I-V curves during reverse Na/K exchange (Bahinski et al., 1988) should provide important additional clues.

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